HETEROGENEITY OF FUNCTION IN HUMAN CD4-6- T-CELL CLONES FROM THE PERIPHERY

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May 9, 1988

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HETEROGENEITY OF FUNCTION IN HUMAN CD4$^-$8$^-$ T-CELL CLONES FROM THE PERIPHERY

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Lisa Ann Straus

1988

ACKNOWLEDGEMENTS

I gratefully acknowledge the teaching and examples set by my advisors Dr. Leonard Chess and Dr. Fred Kantor. In the laboratory I was guided and helped by Dr. Ilan Bank, Judith Cassimeris and Dr. Linda Rogozinski. This thesis would not have been accomplished without the encouragement and computer expertise of my husband and family.

ABSTRACT

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The T-cell receptor (TCR) is the fundamental protagonist in the human immune system, in that it recognizes foreign antigen and initiates the immune response. An antigen is recognized in the context of self major histocompatibility (MHC) encoded molecules on the surface of antigen presenting cells. The capacity for dual recognition of both self and antigen, essential to healthy immune function, is thought to be aquired in the thymus by developing T-cells.

Recently, another rearranging gene, similar to immunoglobulin genes, and specific for T-cells has been discovered and is termed $T\gamma$. $T\gamma$ mRNA appears early in thymocyte development and is postulated to have a role in the selection of T-cells specific for recognition of self MHC encoded molecules. The central questions about the newly discovered receptor pertain to its function in the immune system and its relation to the Ti$\alpha\beta$ TCR.

We used negative selection with monoantibodies to isolate CD4-8- cells from healthy adult peripheral blood and performed functional assays on clones from one individual. Phenotypically the clones are immature: they do not express the Ti$\alpha\beta$ TCR, nor do they express the CD4 or CD8 markers which distinquish T helper and T suppressor cells respectively. The clones demonstrate a heterogeneous cytotoxic response to different targets including K562, T-cell tumor lines, and B-cell lines. In helper cell assays two of the clones show the capacity to induce B-cell activation. This indicates that the $T\gamma$ receptor is functional in the periphery and represents a cell line distinct from both Ti$\alpha\beta$ bearing cells and cells of NK lineage. The cytotoxic and inducer capacity of these $T\gamma$ clones is not MHC restricted, but does demonstrate clonotypic specificity not seen before in $T\gamma$ expressing cells.

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ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
B	Bonemarrow derived
C	Constant
CTL	Cytotoxic T-lymphocyte
DN	Double Negative, CD4-8-
DNA	Deoxyribonucleic acid
E	Erythrocyte
E:T	Effector: Target
EBV	Epstein-Barr virus
Fc	Heavy chain portion of immunoglobulin
G/M FITC	Fluoroscein-conjugated goat anti-mouse immunoglobulin
HLA	Human major histocompatibility complex
IgG	Immunoglobulin G
J	Joining
K	Kilodalton
Kb	Kilobase
mAb	Monoantibody
MC	Miniclone
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
mRNA	Molecular ribonucleic acid
NK	Natural Killer
PHA	Phytahemmaglutinen
PWM	Pokeweed mitogen
RBC	Red blood cell
rIL-2	Recombinant interleukin-2
SRBC	Sheep red blood cell
T	Thymus derived
TCR	T-cell receptor
V	Variable

NOMENCLATURE

The T-cell differentiation antigens detected by monoclonal antibodies are named according to the cluster of differentiation (CD) approach to leukocyte typing.*

Name Used	Human	Mouse
CD1	T6, Leu 6	TL
CD2	T11, Leu 5	
CD3	T3, Leu 4	
CD4	T4, Leu 3	L3T4
CD5	T1, Leu 1	Ly-1
CD8	T8, Leu 2	Ly-2,3
T5		
T9		
T10		
TCR, Tiαβ	CD6	

*Berhard, A. et al. eds., "Human Leukocyte Differentiation Antigens Detected by Monoclonal Antibodies," *Leukocyte Typing* (Berlin: Springer-Verlag, 1984), 9-121. Table adapted from "Note on Nomenclature," *Nature* **325** (1987):660.

INTRODUCTION

The human immune system is a complex regulation of responses to a potentially infinite array of foreign invaders and changes in the host organism. There must be the capacity to recognize foreign antigen either soluble or cellularly incorporated, initiate an effective response, and then regulate that action such that only the target is eliminated. For efficient protection it is necessary that over a lifetime an organism develops a memory for and quick response to some antigens and a tolerance for self.

The cells involved in specific recognition of foreign antigen are bone marrow derived (B) and thymus derived (T) lymphocytes. The B and T cells respond to circulating and cellularly bound antigen, respectively. The T cells are the regulatory arm of the system: able to induce or supress B cell activation, as well as carrying out effector responses to intracellular and cellular signals.

T lymphocytes are activated by recognition of antigen in the context of major histocompatibility complex (MHC) coded cell surface glycoproteins, or by recognition of foreign MHC molecules, on any cell surface.[1] In the first instance there is a dual recognition of self and of antigen and recognition of one or the other is insufficient for activation.[2] This phenomena of recognition is termed 'MHC restriction' because a response to the antigen can only be initiated if the antigen is presented on a cell which also has the MHC antigen of the host organism,[3,4]

There are several possible purposes for this restriction a) to prevent autoaggression when no antigen is present, b) to ensure a swift response to foreign cellular antigens and c) to focus T cell action on the cell

surface rather than upon soluble antigen.[5] The MHC markers which are identified by T-cells are unique to an individual. Class I histocompatibility molecules are expressed on all cell surface membranes and class II histocompatibility molecules are primarily expressed on lymphoid cells. The different MHC molecules are recognized by subsets of T-cells, distiquished by cell surface molecules, CD4 and CD8.[6,7] The $CD4^+$ T-cells recognize class II MHC encoded molecules on other lymphocytes and function as helper cells. The $CD8^+$ lymphocytes recognize class I MHC encoded proteins on other cells and function as cytotoxic or suppressor T-cells.

The simultaneous recognition of antigen and MHC molecule is performed by the T-cell receptor (TCR).[8] The question of whether this molecular complex is two different receptors or a single receptor with dual specificity has been debated[9] with opinion tending towards the latter description. Discussion of Pernis' proposal of one and a half receptors[10] shall be considered later.

Inherent in each T-cell receptor structure is the capacity to recognize a specific antigen when properly presented. Considering the unlimited possible antigens to be encountered, the system must be able to generate an extremely diverse range of antigen specific receptors. The diversity required is generated by several mechanisms including DNA rearrangements and somatic mutations.[11] The expression of unique combinations of variable (V), joining (J), and constant (C) gene regions serves to generate the panoply of receptors needed.[12] The capacity to transpose gene segments in order to generate unique peptide sequences in a conserved structure is characteristic of the immunoglobulin super-

gene family.[13] The genes for the V, J, and C regions of the TCR are similar in size and sequence to those which encode the immunoglobulin proteins.[14,15,16,17]

The TCR is a disulfide linked 90 K heterodimer (Ti), composed of α, a 48-54 K acidic molecule, and β, an electrophoretically more basic 40-44 K subunit.[18] These transmembrane glycoproteins each have variable and constant regions encoded by genes of the immunoglobulin supergene family.[19,20] The TCR is noncovalently associated with the CD3 molecule.[21] The CD3 molecule is composed of three noncovalently linked, nonpolymorphic peptides (CD3γ 25K, CD3δ 20K, and CD3ε 20K).[22,23] (Figure 1) Evidence to support the linkage of Tiβ and CD3 includes the stoichiometric relation of CD3 and Tiαβ and the ability to coimmunoprecipitate the two molecules.[24,25] The CD3 molecule is thought to function in transmembrane signaling events related to TCR activation.[26] Perturbation of the CD3 molecule can induce or inhibit TCR activation,[27] and leads to an increase in cytoplasmic free Ca^{++}.[28] Binding of anti-CD3 mAb can cause comodulation (via endocytosis[29]) of the TCR, or serve as an agonist producing T-cell activation.[30] Incubation with a low concentration of mAb to CD3 induces proliferation, lymphokine production, and cytolysis while higher concentrations of anti-CD3 inhibit these functions.[31,32]

In order to learn more about T-cell function and the TCR investigators have explored thymocyte development.[33,34] Prothymocytes migrate from the bone marrow to the thymic cortex. The 'early' stage of T-cell ontogeny is identified as that 10% of thymocytes which express T9, T10 and CD2. The 'common' stage cells, representing 70% of thymo-

cytes, also in the thymic cortex, express CD4, CD8, CD1, T5, T10 and CD2 markers concurrently. In the process of maturing the thymocytes lose the CD1 antigen and differentiate into either $CD4^+8^-$ or $CD4^-8^+$ subgroups. In the final stage of development, which takes place in the medulla of the thymus and includes 10% of all thymocytes, the cells aquire the Ti/CD3 complex and are prepared to circulate in the periphery.[35] The mature thymocyte has a TCR/CD3 complex T12, CD2, CD5 and either a CD4 or CD8 marker. The T10 marker is lost when the T-cells migrate to the periphery but does appear on 10% of non T-cells in normal adult blood. (Figure 2) Umiel's research in T-cell ontogeny revealed a population of immunocompetent $CD3/TCR^+$ cells in the thymic cortex.[36]

In order to trace TCR development specifically, investigators have delineated the order of appearance of the gene transcripts for Tiα and Tiβ in humans[37,38,39] and in mice[40,41,42]. Gene transcripts for the Tiβ are present at the earliest stage of human thymic development in the form of an incomplete 1.0 Kb transcript for the Tiβ D-J-C region. The Tiβ transcripts are found in greatest number during the intermediate or common stage of development in the cortex. This is in contrast to the Tiα which is undetectable in the earliest stage of development and only reaches peak production when the cells are in their final stage of maturation. This discrete order of expression parallels the transcription of immunoglobulin genes.[43]

In pursuit of the 1° structure of the Tiα, Saito, et al. examined the cDNA from murine alloreactive CTLs and found a rearranged and expressed gene which they postulated was a second T-cell receptor.[44,45]

They discovered that the gene included variable (V) and constant (C) domains with distinct homology to the immunoglobulin genes and cysteine residues in position for the formation of S-S bonds in the peptide. This third rearranging gene (after Tiβ and Tiα), unique to T-cells, is called T_γ. Krantz[46] and Hayday[47] continued to elucidate the capacity of the gene to generate diverse receptor regions and they noted that the mRNA is found primarily in cytotoxic rather than helper T-cells. Later studies found that five V regions nonrandomly crosshybridize with $C_\gamma s_{1-4}$ and these recombinations are variably found in immature and mature T-cells.[48,49,50]

Raulet noted that large amounts of T_γ mRNA are expressed in fetal thymocytes as compared to very little in adult cells.[51] During thymocyte maturation first T_γ is expressed, then Tiβ, and finally Tiα.[52,53] Noting that most thymocytes die in the thymus and that T_γ mRNA is present at a specific time in ontogeny Raulet postulates that this new receptor is instrumental in the selection of thymocytes that will be MHC restricted. In other words, the population with this receptor is active in the thymic education of T cells proposed by Zinkernagel[54] and Bevan[55]. Another suggestion is that the new receptor recognizes the MHC antigen in conjunction with the recognition of antigen by the traditional TCR, i.e. dual recognition with two receptors functioning. The latter idea is less likely because the T_γ molecule itself has not been isolated and the genetic evidence for it is not common in normal adult cells.

The T_γ gene has been investigated in humans[56,57] and has been shown to have six V, two J, and two C regions. The constant gene regions are localized to the short arm of chromosome 7 and display much

homology to the corresponding murine genes. One of the C_γ region rearrangements is deleted in the T_γ expressing cells examined which could be due to a translational frame shift,[58] or a regulatory mechanism which determines cell lineage as proposed for leukemic cell lines.[59] LeFranc[60] noted that the $J_{\gamma 2}C_{\gamma 2}$ generates a protein without cysteine residues for disulfide linking.

In the continuing search for the T_γ molecule Bank[61] isolated and cloned thymocytes which were $CD4^-8^-$ (Double Negative-DN) and $CD3^+$. Northern blots for this cell line showed T_γ mRNA as well as 1.3 Kb message for mature Tiβ, 1.0 Kb D-J segment of Tiβ, and an immature 1.4Kb Tiα transcript. Immunoprecipitation with anti-CD3 antibody revealed a novel nondisulfide linked heterodimer of 44Kb and 62Kb proteins (instead of the 40 and 49Kb of the Tiαβ). When anti-CD3 mAb was used as an agonist to activate the clone with the potential new receptor, it killed K562, a classic target of cytotoxic lymphoytes. A phenotypically and genetically immature cell line was shown to have a molecule other than the known TCR associated with CD3 and the capacity to function as a mature T cell.

The fact that the putative new receptor T_γ is found on functional T-cells is extremely important. This is a step in the definition of the T_γ receptor, and begins to address the question of how this receptor relates to the more common TCR. Further study is required to answer questions about the purpose of the T_γ receptor and the function of T-cell receptors in general. Formerly identified genetically, by looking for mRNA production, cells expressing the T_γ may now be isolated according to function. This brings us one step closer to actually analysing the

molecule which has so far proved elusive. It will be necessary to consider lymphocyte function and cytotoxicity in particular, in order to pursue knowledge of the T_γ receptor and the cell lines expressing it.

Cytotoxicity,[62] was recognized in vivo as allograft rejection and then in vitro in mixed lymphocyte cytotoxicity (MLC). The cytotoxic T lymphocyte (CTL) requires sensitization of the effector cell to the target cell over a period of 7-14 days, is MHC restricted, and usually expresses the T8 marker. Investigators noticed that some cytotoxic T lymphocytes were able to lyse targets without prior exposure. These are natural killer (NK) cells which Herberman identifies as large granular lymphocytes which kill tumor cells, transformed or infected cells without prior immunization.[63] Natural killer activity is not MHC restricted. The standard for NK activity is the ability to kill K562, an erythromyeloid cell line without MHC class I or class II molecules on its surface. A subset of NK cells have Fc receptors and lyse targets which have been coated with antibody. Molecules on the NK cell surface bind to the tails of antibodies bound to a target cell bringing the two cells into close proximity and facilitating the subsequent killing of the target cell. This ability is termed antibody dependent cellular cytotoxicity-ADCC. Hercend, et al. were able to expand our knowledge of human NK cells by showing that clonal lines were both phenotypically and functionally heterogeneous.[64] Some cells which have NK activity are $CD3^+$ and some are $CD3^-$ while all are $CD2^+$ and most are $CD8^+$. Cell lines with similar phenotypes have different ranges of cytotoxicity. It has been noted that the cytotoxic function of a $CD3^+$ line can be inhibited by incubation with anti-CD3 antibody.[30] Lanier[65] focussed on

the $CD3^+$ cells with NK function and observed that this subset is 2-5% of all lymphocytes, has an increased level of CD3 expression relative to other T-cells, and is Fc receptor positive, i.e. capable of ADCC. Initially, these cells had low anti-CD8 reactivity, but after three weeks the population was 80% $CD4^-8^-$. The selected population was only cytotoxic in the presence of anti-CD3 or IgG2a coated targets, and in these cases it was not antigen specific and did not require MHC recognition. Lanier identified a population of $CD3^+$, Fc^+, non MHC restricted CTL which are $CD4^-8^-$.

To further explore these possible precursors of CTL, De La Hera used negative selection with mAb and complement to isolate a $CD3^+4^-8^-$ population of human thymocytes from children and showed that the cytotoxicity to K562 can be inhibited using mAb to CD3.[66] Lanier isolated a population of these double negative (DN) cells from normal peripheral blood and noted that their cytotoxic response to K562 was significantly augmented by the presence of anti-CD3.[67] The seemingly opposed effects of the anti-CD3 may be due to different concentrations of the mAb used. Lanier and Bank have each isolated a cell population with a unique combination of phenotype and function. Do cells with the DN phenotype represent a stage in differentiation or a distinct cell line? What receptor is operating to allow these cells to function? What can we learn about the mechanism and purpose of this type of cytotoxicity? Could this function have a role in thymocyte development? At this point we know that T-cells which express the potential new receptor are phenotypically immature and have cytolytic function.

Investigators are exploring several new population sources for cell lines which are phenotypically and functionally similar to those thought to express the $T\gamma$ molecule. Brenner[68] isolated a DN population from the blood of an immunodeficiency patient, which did not have transcripts for the $Ti\alpha$ or $Ti\beta$ components of the TCR, but did have $T\gamma$ mRNA. Immunoprecipitation with anti-CD3 revealed a nondisulfide linked heterodimer composed of 55K and 40K peptides. Antibody to synthetic $T\gamma$ proteins also precipitated the 55K molecule. He postulated that this heterodimer was $T\gamma\delta$ or $T\gamma\gamma$ where one $T\gamma$ was differently glycosylated.

Weiss characterized the phenotype and genetic complement of Peer, a leukemic T-cell line.[69] The cells are $CD1^-2^-3^+4^-8^-WT31^-$ and have low levels of $Ti\beta$ but no $Ti\alpha$ transcripts. (WT31 is a monoantibody to a component of the TCR.[70]) Immunoprecipitation identifies a nondisulfide linked 55K molecule, which is either a hetero or homodimer, associated with the CD3 molecule. Nowill[71] and Moingeon[72] et al. have isolated clones from fetal (25 wk old) blood which are $CD3^+4^-8^+WT31^-$ and cytotoxic to K562, Molt4, and JM. These cells have mRNA for 1.0 and 1.3Kb $Ti\beta$ but not $Ti\alpha$ on Northern blots. Immunoprecipitation with anti-CD3 mAb or with a clonotypic mAb demonstrated a 41/44K disulfide linked hetero or homodimer. The clonotypic mAbs inhibited the cytotoxicity of the clone.

If functional $T\gamma$ expressing cells are an immature form of T-cell then one can expect to find them in the thymus, and immunodeficient, leukemic, and fetal blood. However, if we are characterizing a distinct cell line then evidence of this population may be found in normal adult peripheral blood. Perhaps the phenotypically immature cytotoxic T-cells

isolated by Lanier correspond to $T\gamma$ expressing cells. Further characterization of the chemical and genetic nature of the $T\gamma$ protein as well as its functional capacity is required to answer these questions.

Borst[73] and Brenner[74], in separate studies, examined DN clones from normal human peripheral blood. Some of these were found to be $CD3^+2^+WT31^-$ and to have $T\gamma$ mRNA but no Tiβ or Tiα by Northern blot and immunoprecipitation studies. The $T\gamma$ receptor expressed exists predominantly as a disulfide linked heterodimer of variously weighted γ and δ chains or perhaps a homodimer with the subunits differently glycosylated. This is in contrast to the nondisulfide linked $T\gamma$ receptor seen on Peer and IDP2 cell lines. It is hypothesized that the difference in linkage of $T\gamma$ residues on human peripheral blood lymphocytes (PBL) depends upon which constant region, $C\gamma_1$ or $C\gamma_2$, is utilised[75]. The $C\gamma_2$ gene segment does not have cysteine residues and therefore leads to production of a nondisulfide linked homo or heterodimer.[60] Murine $T\gamma$ and human Ti$\alpha\beta$ are invariably disulfide linked. Further study of the TCR receptor genes is needed to answer questions concerning the chemical and genetic nature of the $T\gamma$ expressing cells and their relation to the Ti$\alpha\beta$ expressing cells.

T-cells in the periphery, which do not have functional Ti$\alpha\beta$ and do express $T\gamma$, have been shown to have non-MHC restricted cytotoxic capacity. The $T\gamma$ receptor, similar to the mature Ti$\alpha\beta$ receptor, is associated with the CD3 molecule and perturbation of that molecule alters cytotoxic function. Moigeon, et al.[72] were able to further demonstrate that the $T\gamma$ receptor itself is functional using a clonotypic mAb which inhibited cytotoxicity of a clone from fetal blood.

Our goal is to understand human T-cells and their function. Specifically, how is their action mediated by the TCR? The newly discovered receptor brings many questions to light. What role does the $T\gamma$ receptor play in the T-cell immune repetoire? What is the range of function of this receptor? Though these DN populations lack the traditional T-cell antigens associated with helper and suppressor activity perhaps they are capable of performing other T-cell functions beyond cytolysis. The $T\gamma$ receptor as it is currently known differs from the Ti$\alpha\beta$ in that its functions are not MHC restricted. Is there any antigen specific or MHC restricted cytotoxicity as yet undetected?

In order to explore the nature of the $T\gamma$ receptor as posed in the questions above, we have generated DN clones from normal human peripheral blood. These cells have the CD3 protein but lack the Ti$\alpha\beta$ molecule on the cell surface. Previous studies[73,74] have indicated that cells of this phenotype express the $T\gamma$ molecule. Cells from normal human adult peripheral blood have been postulated to represent a mature stage of the $T\gamma$ cell and are, therefore, the best candidates for a study of the range of function of this receptor. Comparison of a number of different clones from one individual offers the opportunity to examine the question of receptor specificity. The $CD3^{+}4^{-}8^{-}WT31^{-}$ clones in this study are shown to have heterogeneous specificity with regard to non-MHC restricted cytotoxicity and helper function.

MATERIALS AND METHODS

Isolation of CD4-8- T-cells

Blood was obtained from healthy adults and viable T lymphocytes were separated using Ficoll-Hypaque density gradient centrifugation and sheep red blood cell (SRBC) (Colorado Serum) rosettes[76]. The T-cells at 20 x 10^6 cells/ml were incubated in OKT4 (1/1000) and OKT8 (1/2000 final dilution) for 45 minutes at 4°C and then rosetted to ox RBC (Colorado Serum) coupled to rabbit anti-mouse IgG at a final hematocrit of 2.5%. The CD4-8- cells which were not rosetted to the ox RBC were separated out using Ficoll-Hypaque density gradient centrifugation.

Culture

CD4-8- cells were cultured at 1 x 10^6 cells/ml in the presence of 1 x 10^6 cells/ml of an irradiated (6750 rads) allogeneic B lymphoblastoid line in 1.5 ml in 24 well tissue culture plates (Costar). The culture was stimulated with rIL-2, 100u/ml (Hoffman LaRoche), and 1% phytahemmagluttinen (PHA) (Gibco). The final medium for all cultures is Iscove's modified Dulbecco's medium (Gibco) supplemented with 5% fetal calf serum (Hyclone), 1% penicillin-streptomycin (Gibco), and 1% glutamine (Whittaker). On day 10, the bulk culture was 'minicloned' at 10 cells/well in 96 well U-bottomed plates (Costar). These cultures were maintained with rIL-2, 100u/ml, 2X/week, and 0.4 x 10^6 cells/ml of irradiated feeder cells, as described above, 1X/week, and were grown in a humidified atmosphere with 5% CO_2 at 37°C.

Cytofluorographic Analysis

1 x 10^5 cells were incubated with the appropriate antibodies (anti-CD3 hybridoma supernatant 1:4, OKT11 1:500, OKT4A 1:250, OKT8 1:500, and WT31 1:1000 final dilution) at 4°C for 30 minutes. The cells were then washed and incubated in the fluoroscein-conjugated goat anti-mouse immunoglobulin (G/M FITC) (Meloy Laboratory) 1:100 final dilution at 4°C for 45 minutes and washed. Fluorescence was analysed on a model 30-H cytofluorograph (Ortho instruments). The results represent analysis of 10^3 cells for each population as indicated.

Cytotoxicity

The target cells: K562, U937, Molt4, Frolich, and B-Lymphoblastoid lines were grown under culture conditions as described above. Autologous and allogeneic resting E^+ and E^- lymphocytes were obtained using Ficoll-Hypaque density gradient centrifugation and overnight rosette with a 5% SRBC solution. E^+s were separated from SRBC with Tris-buffered ammonium chloride. Cells were maintained in medium at 37°C in a humidified atmosphere with 5% CO_2 for 48 hours. 0.5 x 10^6 cells of each target population were treated with 24uCi ^{51}Cr-chromium sodium (specific activity 200-500 uCi/mg of Cr) (New England Nuclear), incubated for 1 hour at 37°C, washed and placed at 5000 cells/well in 96 well U-bottomed plates(Costar). Controls were the target cells alone and target cells plus 100ul 10% Triton solution in a final volume of 200ul. The effector cells were added at the indicated E:T ratios in a final volume of 200ul. Where indicated, the effector cells were incubated with an anti MHC Class I mAb, W6/32[77], (anti HLA-A, B, C

monomorphic determinant) (1:400) or anti-CD2 (1:400) for 30 minutes at 4°C (before being added to the targets). After 5 hours incubation at 37°C in a humidified atmosphere containing 5% CO_2, 100ul of spun supernatant was removed from each sample and the ^{51}Cr content measured. Specific cytotoxicity was calculated as follows:

(Experimental ^{51}Cr release - Spontaneous ^{51}Cr release)/(Total ^{51}Cr release - Spontaneous ^{51}Cr release)

Total ^{51}Cr release was assessed by Triton lysis of the ^{51}Cr-labelled cells, spontaneous ^{51}Cr release was assayed in target cells alone.

Proliferation

5 x 10^4 DN cells were added to 5 x 10^4 resting E^+ or E^- cells, obtained from normal adult blood as described above and irradiated (2250 rads) in a final volume of 200ul of medium in 96 well U-bottomed tissue culture plate (Costar). Assays were performed in triplicate. The plates were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 0, 3, and 6 days of culture, cells were harvested in a Mash II apparatus and the amount of ^3H-thymidine incorporated over a 16 hour period was measured as counts/minute in a liquid scintillation counter.

Reverse Hemolytic Plaque Assay

In order to isolate a B-cell population from resting E^-lymphocytes, the cells were incubated at 4°C for 20 minutes in anti-CD3 hybridoma supernatant (1:20 final dilution), washed, agitatated in rabbit complement (1:10) at 37°C for 1 hour, and then allowed to adhere to a large petri dish (Nunc) at 37°C for 1 hour. 2.5 x 10^4 B-cells were added to each tube and autologous T-cell populations at 10%, 50% and 100% of the

B-cell number were added as indicated. Pokeweed mitogen (Gibco) was added at a final dilution of 1:200 to appropriate samples. The final volume was 1 ml for each tube. Cells were incubated in culture conditions previously described for 7 days. Plaque assays were performed by adding 10ul each of 11% SRBC coupled to rabbit anti-human IgG, guinea pig complement (1:2), and rabbit anti human IgG (1:100) to 50 ul cell suspension and suffusing the mixture into a chamber formed of taped microscope slides. This was incubated at 37°C for 1-2 hours and the plaques were counted. Plaque forming capacity equals the number of plaques/10^6 B-cells.

RESULTS

Initial Study of Peripheral DN

$CD4^-8^-$ cells were isolated from normal human peripheral E^+ cells using negative selection with OKT8 and OKT4 mAb. This population is approximately 1-3% of the total circulating T-cell population. These cells did not react with WT31, a framework mAb to the TCRαβ heterodimer, but were shown to be $CD3^+$ upon cytofluorographic analysis. Immunoprecipitation with mAb to CD3 or Tγ antisera revealed a 75K peptide which under reducing conditions resolved into a heterodimer of 40K and 36K. The Tiβ and Tiα peptides were not co-precipitated from the cell surface.

In order to discover whether cells which do not have the Tiαβ TCR and do express T_γ mRNA perform known T-cell functions, cytotoxicity

assays were performed. The peripheral DN cells demonstrated potent cytolytic activity towards K562 at a 20:1 effector to target ratio. They were spontaneously cytotoxic to K562 (84%), U937, a monocyte, (72%), an allogeneic B cell line, DR8, (23%), and autologous E^- cells which were incubated overnight with 10% PWM and 50% irradiated E^+, (53%). (Figure 3) Examination of the phenotype during the same period showed that the cells continued to be $CD3^+4^-8^-WT31^-$.

Phenotype of Clones from Peripheral Blood

The isolation of a $CD4^-8^-$ population of T cells from normal human blood was repeated and again the initial phenotype depicted a $CD3^+4^-8^-WT31^-CD1^-$ population. The culture was started with 1% PHA and was maintained with Il-2 and feeder cells as above. In order to avoid the possibility that a subpopulation of NK cells was functioning the cells were 'minicloned' at 10 cells/well and subsequently 19 cell lines were maintained separately. The initial phenotype of these cells showed that they were all $WT31^-$ and that all but #19 were $CD3^+$. All lines were $CD2^+$. Phenotypes repeatedly performed alongside functional experiments demonstrated that the populations tested maintained stable profiles of $CD3^+4^-8^-WT31^-$ and $CD2^+$ with the exception of #19 which continued to be $CD3^-$.

In the periphery there is a heterogeneous population of $CD2^+4^-8^-$ cells which can be divided into those with and those without CD3. It is most interesting that those clones which react to anti-CD3 antibody appear to be without the Ti$\alpha\beta$ which was formerly thought to be ex-

pressed in conjunction with CD3 after differentiation into $CD4^+$ and $CD8^+$ populations.

The miniclones were later found to express $T\gamma$ molecules which were coimmunoprecipitated with CD3. The clones did not express immunoprecipitable $T\alpha$ or $T\beta$. (Personal communication from Dr. Ilan Bank)

Cytotoxicity

In previous studies, phenotypically 'immature' cells with cytotoxic capacity, were isolated from thymus[30]; blood of an immunodeficiency patient[42], a leukemic patient[43],and a fetus[44,45]. These cell lines were postulated to represent a stage in thymic differentiation. Recent studies have shown that phenotypically similar populations isolated from the periphery also have cytotoxic function and may well represent a mature $T\gamma$ population.[46,47] In our preliminary study, we confirmed that the DN T-cells from the periphery are non-MHC restricted cytotoxic lymphocytes. In order to further characterize this effector function, we compared a series of clones of peripheral DN cells according to their cytotoxic function.

Target Range

Peripheral blood lymphocytes, with the DN phenotype, at an effector to target ratio of 20:1, are spontaneously cytotoxic to K562, a cell line derived from an erythromyeloid precursor and notable for the absence of class I and class II HLA molecules. Miniclones MC9, MC11, and MC12 were tested repeatedly and they demonstrated a capacity to kill at between 5-34% never reaching the levels of MC19 which was consistently

cytotoxic to K562 in the 70-80% range. MC9 generally had low to negligible levels of cytotoxicity to K562. One miniclone, hereafter MC4, was not, in repeated trials, cytotoxic to K562.

Molt4, an immortal T-cell line, was also subject to killing by all of the peripheral DN clones except MC4, which was ineffective in 2 out of 3 assays. (Figure 3.1) Similar data was obtained for another T-cell tumor line, Frolich, where MC4 had 0% cytotoxicity and MC11 lysed 14% of the target cells (data not shown).

These cell lines were also repeatedly tested for cytotoxicity on U937, a monocytic cell line notable for having many Fc receptors. All of the miniclones were cytotoxic to U937 with MC8 having the highest levels with 39% cytotoxicity and MC9 the lowest at 9%.

Continued cytotoxicity target range assays at an effector to target ratio of 20:1 revealed that MC4 was the only line which consistently was cytotoxic to its feeder B-lymphoblastoid cell line, BA. (Figure 3.2) Further studies showed that MC4 killed four other EBV immortalised B-cell lines including one (data not shown) begun from the autologous donor. The other clones; 9,11,and 12 had little to no cytolytic activity on these same cell lines. (Figure 4).

The MCs 4, 9, 11, and 12 were, in general, less cytotoxic to targets than MC19 which is $CD3^-$. Where MCs 9, 11, and 12 lyse K562, Molt4, U937 and Frolich to varying degrees, MC4 is only effective on U937 and immortalised B-cell lines including autologous B-cells. The possibility that MC4 is specific for HLA Class II or an EBV determinant is less than likely because it is also cytotoxic to U937. The peripheral DN clones are heterogeneous in their specificity. MC4 has a distinctly

different cytotoxic specificity from the other miniclones while maintaining the same phenotype.

Non-MHC Restricted Cytotoxicity

The clones appear to be non-MHC restricted in that they have the capacity to lyse cells which do not have class I or class II antigens (K562). This may be a nonspecific toxicity which coexists with a capacity for a specific response dependent upon MHC antigen recognition. In order to examine this we used autogeneic and allogeneic cell lines as targets. Miniclones 9,11, and 12 were not at all cytotoxic to allogeneic or autologous resting E^+ or E^- cells which had been incubated for 48 hours before the assay. MC4 was only tested on autologous E^- cells and the results were inconclusive due to poor uptake of chromium by the targets.

In an attempt to elucidate any possible role of the class I antigen in the recognition of the target we tested the cytotoxicity of clone 4 and clone 11 on the feeder cell line, and on K562 respectively after incubation of the target with W6/32, an anti-MHC class I mAb. The cytotoxicity of MC4 was greatly enhanced which was probably due to ADCC and should be repeated with blocking of Fc receptors. The cytotoxicity of MC11 was unchanged by the addition of W632 to the target. This would have to be repeated over a dose response curve to show that this was not just a case of being at a plateau in the curve.

Proliferation

In further pursuit of the question of MHC restriction, we asked whether the DN miniclones would proliferate in response to autologous irradiated E^-s? In repeated experiments, though E^+ controls proliferated well in response to allogeneic E^-s and slightly to autologous E^-s on days 3 and 6, none of the miniclones did so in response to either auto or allogeneic E^-s.

Helper Function

We also wanted to know if these $CD4^-$ cells could perform helper function and if so would there be any heterogeneity amongst the clones as seen in the cytotoxicity assays? MCs 4, 9, 11, and 12 were incubated with autologous resting B cells (isolated using anti CD3 and adherence) at ratios of 1:10, 1:2 and 1:1 of T-cell to B cell, with and without PWM, and assayed for plaque forming capacity on day seven. Without PWM there was no evidence of experimental inducer function. It is clear from the replicated results that in the presence of PWM both clone 9 and 11 are able to function as helper cells and that clones 4 and 12 do not. (Figure 5) It is possible that clone 4 suppressed the B-cell activity. In the presence of MC4 there were fewer plaques formed than in the control which had no E^+ lymphocytes. We have seen (Figure 4) that MC4 is cytotoxic to all the B-cell lines which it has encountered. The question of this clone's cytotoxicity to PWM stimulated B-cells needs to be further tested. It will also be important to further delineate the limits of the helper capacity demonstrated by MCs 9 and 11. Once again

these phenotypically similar clones demonstrate a marked heterogeneity of effector capacity. Cells which do not express CD4, and are also capable of cytotoxicity, can function as inducers of B-cell response.

DISCUSSION

We have selected a $CD4^{-}8^{-}$ population from normal peripheral blood and observed along with Borst and Brenner that these clones are spontaneously cytotoxic to a broad range of non MHC restricted targets.

The fact that the clones examined in this study have different target specificities, i.e. MC4 kills B cell lines and MCs 9,11, and 12 are cytotoxic to T cell tumor lines, supports the idea that this cytotoxicity is receptor mediated. The heterogeneous target specificity of cells which express the $T\gamma$ but not the $Ti\alpha\beta$ receptor, is an indication that the receptor is capable of antigen specificity. Further investigation of the function of the $T\gamma$ receptor will be aided by the use of a mAb to the $T\gamma$ receptor itself.

One observation that arises is that simply by our selection technique we are isolating from the periphery a subset of, and therefore must include, NK cells in our experimental population. In fact, MC19, which is $CD3^{-}$ and is cytotoxic at very low doses to 'classic' NK targets, is probably an example of what Lanier would term an NK cell.[78] The similarities of the DN clones to NK cells include: non MHC restricted cytotoxicity, the ability to perform ADCC, and the lack of need for sensitization to the target of cytotoxicity. Though our data as to the effect of CD3 on the function of the $T\gamma$ receptor is inconclusive, Borst,

Brenner and Moingeon interpreted their data to show that CD3 is functioning on cells with the $T\gamma$ receptor. The presence of a CD3 molecule associated with a receptor serves to distinquish this population from other cells with NK function. The prediliction of one of these cell lines for B-cell lines also serves to distinquish cells of this phenotype from other cells with 'NK like' function. This particular specificity would be very rare for a $CD3^-$ cell line. Another point of difference between T_γ expressing cells and NK cells is that there is a population of T cells found in the thymus which is not the case for those of NK lineage.[79]

Our data reconfirm that this population is cytotoxic to a broad range of cells and does not discriminate between self and nonself. MC4 kills its own activated B-cell line but not autologous E^- lymphocytes. Reinhertz aptly questions the non-MHC restricted quality of DN cytotoxicity.[75] The broad range of targets killed by these clones may be an in vitro phenomenon quite apart from an MHC restricted specificity as yet unmeasured. Our finding that some of the clones (MCs 11 and 9) function as helper cells in an autologous system highlights the fact that further study of MHC recognition by these cells is necessary.

In considering the purpose of this new receptor one must return to the question of lineage. Do different populations of cells with functioning $T\gamma$ receptors represent stages in the development of the $T\gamma$ receptor or is the $T\gamma$, as Raulet et al. assert, itself necessary for the functional maturation of the $Ti\alpha\beta$ receptor? Borst and Brenner postualte that the $T\gamma$ genetic rearrangement takes place in the thymus and that the T_γ expressing cells which are isolated from the periphery are mature

examples of this receptor. In other words Tγ expressing cells are a separate fully differentiated subset of T-cells.

Evidence that the Tγ and the Tiαβ are separate branches of cell lines with a common precursor, perhaps entirely different lineages, or even coexpressed would not preclude the Tγ expressing cells from having a role such as that proposed in the thymic education of the preponderant T-cell receptor. The observation that Tγ mRNA production is increased during fetal life when MHC restriction is thought to be determined makes it a tempting participant in the selection of self recognizing clones. Pernis speculates that dual recognition is accomplished by a 'one and a half receptor' model composed of a complex of αβCD3β where Tiαβ recognizes antigen and βγ recognizes self MHC encoded molecules.[10]

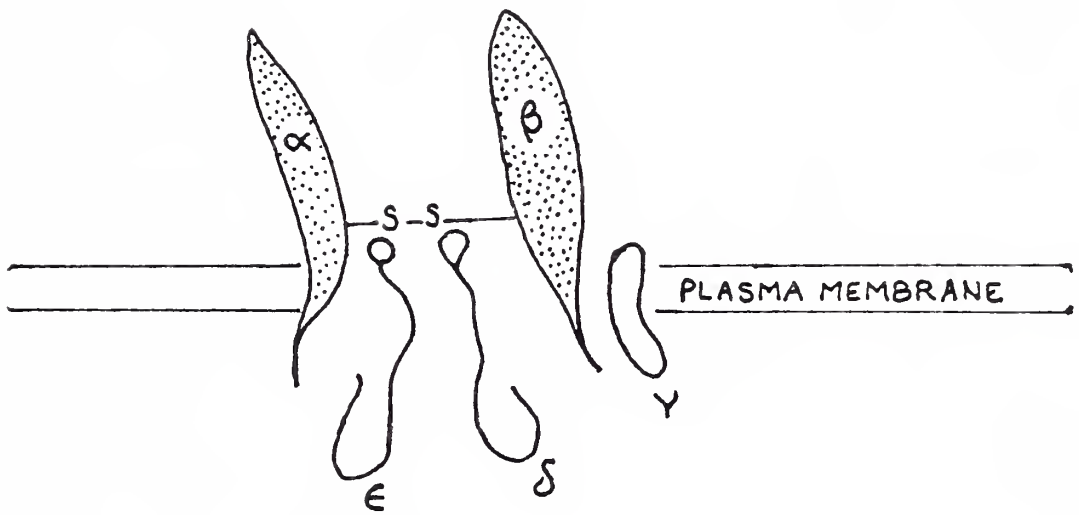
It is also entirely possible, as suggested by Reinhertz and Nakanishi, that the Tγ receptor is simply an evolutionary footnote, with a distinction from the Tiαβ similar to that between the κ and λ light chains.[80] Perhaps the increased level of Tγ mRNA during fetal life is simply ontogeny recapitulating phylogeny. Discovering the genetic lineage and expression of the T-cell receptor(s) in humans is important in giving us clues to the role, if there is one, for Tγ in T-cell development. We have demonstrated that some Tγ expressing cells have helper and cytotoxic function, and that the cytotoxicity itself varies in range from clone to clone indicating that the receptor is capable of some degree of antigen specificity.

In summary, a receptor which is detected genetically in an early stage of T-cell development is found on T-cells from healthy adult

human peripheral blood. The T_γ molecule is expressed in the context of CD3 but without the Ti$\alpha\beta$ TCR. Neither does it appear with CD4 or CD8, the markers of helper and suppressor T-cells. It is possible that T_γ expressing cells represent a distinct cell line with intermediate functional and antigen recognition capacity relative to the Ti$\alpha\beta$ TCR. The purpose, if any, of this receptor is as yet unknown.

FIGURES

Figure 1. Model of TCR/CD3 Complex*

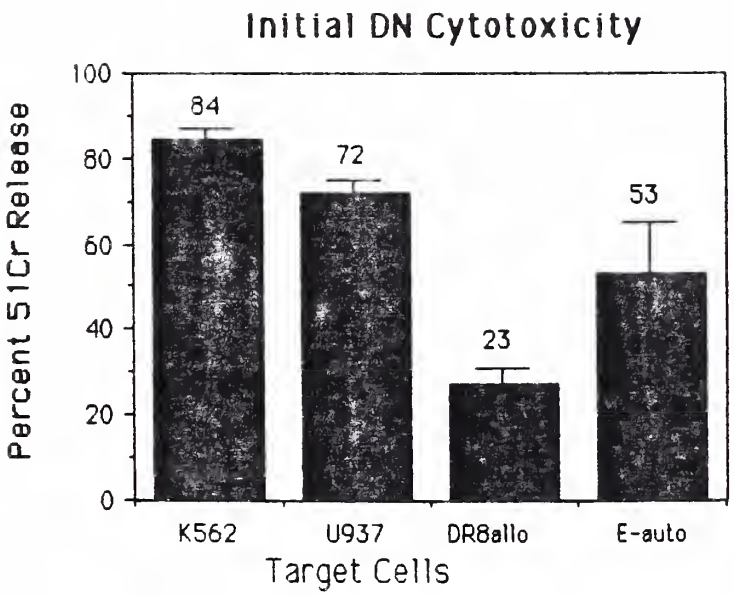


The TCR heterodimer of α (48–54 K) disulfide bonded to β (40–44K) is in a complex with the CD3 molecule composed of γ (25 K), ε (20 K), and δ (20 K). The functional connection between the TCR and CD3 may be mediated by a structural link between Tiβ and CD3γ.[24] *Adapted from Weiss et al.[26]

Table 1. T-Cell Ontogeny

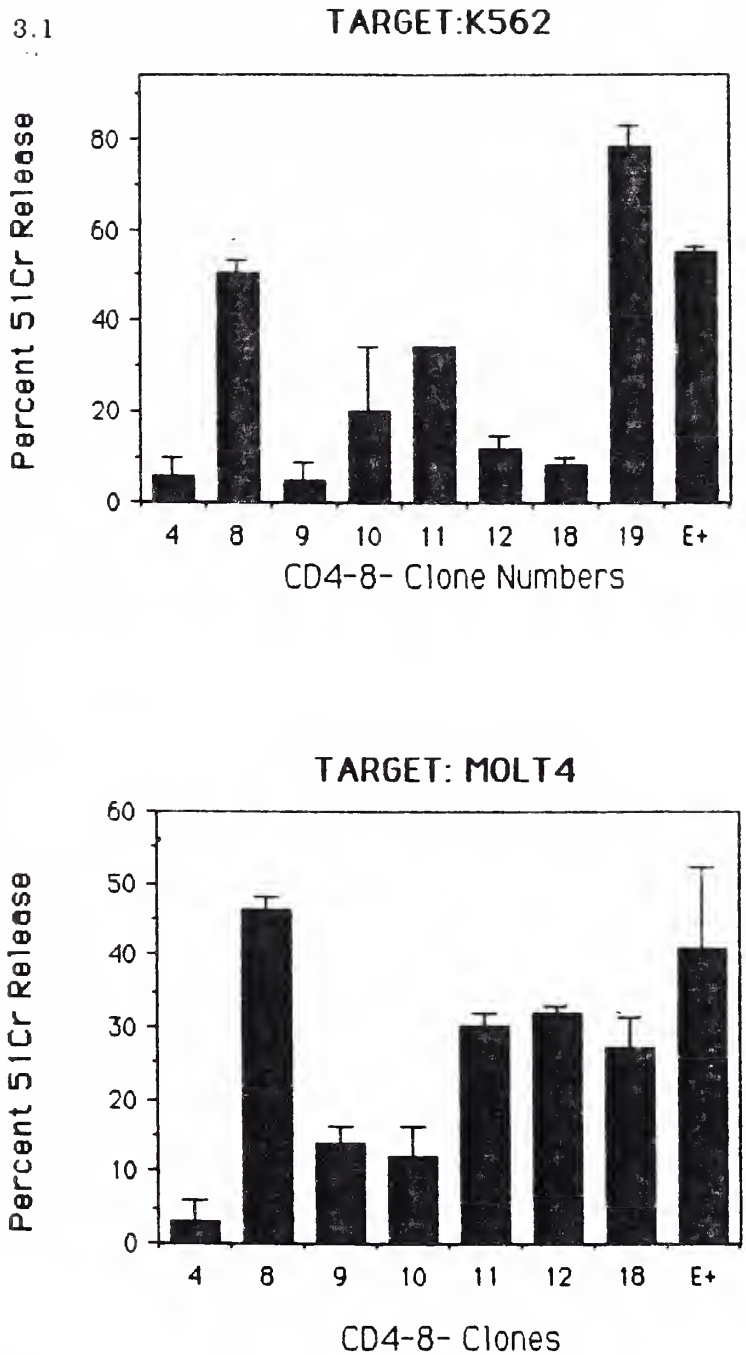
Stage	Location	% Thymocytes	Surface Molecules Expressed		mRNA Transcribed
Precursor	Bone Marrow	___			
Early	Thymic Cortex	10%	T10 T9 CD2		Tiβ +
Common	Thymic Cortex	70%	T10 CD2 CD1 CD4 T5 CD8		Tiβ ++ Tiα +
Mature	Thymic Medulla	10%	T10 CD2 CD3 TCR CD4	T10 CD2 CD3 TCR CD8 T5	Tiβ + Tiα ++
	Periphery	___	CD2 CD3 TCR CD4	CD2 CD3 TCR CD8 T5	

Figure 2.



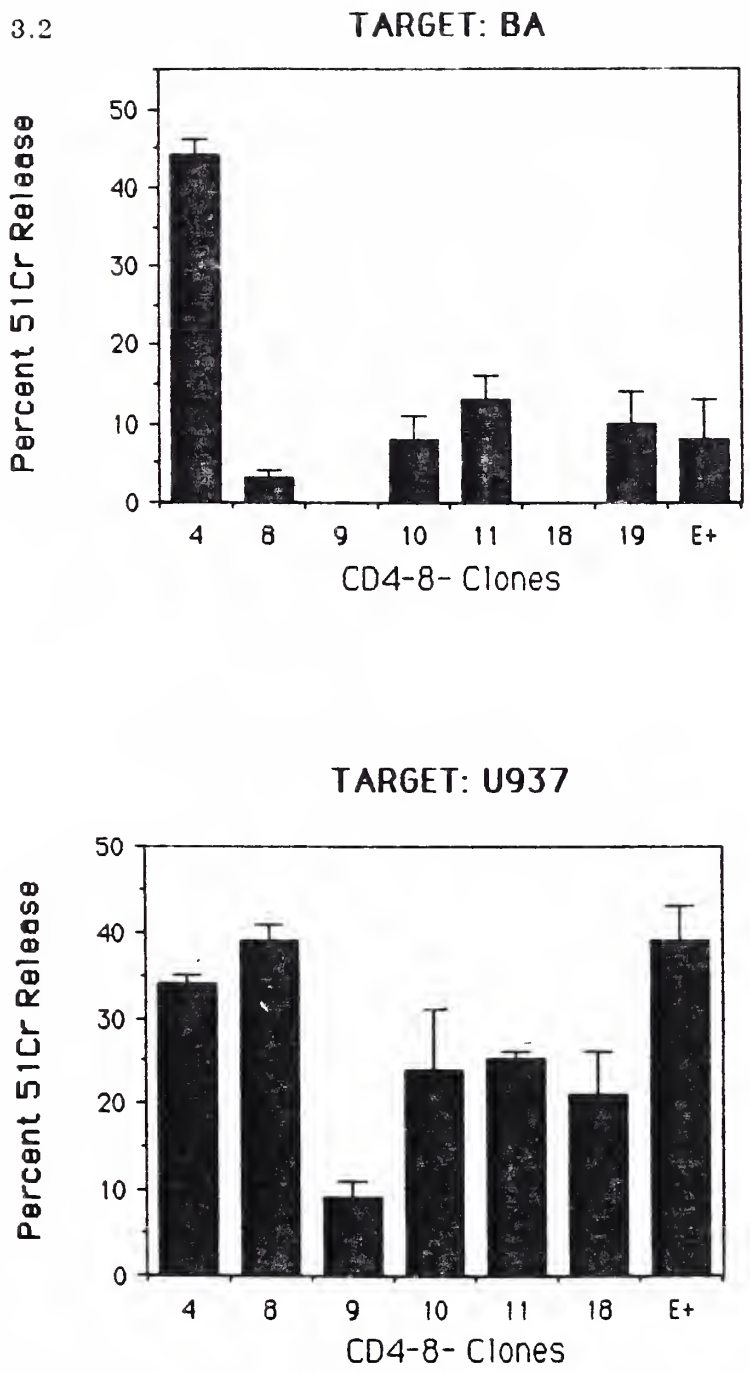
CD3+4-8-WT31- T-cells maintained in culture are cytotoxic to K562, U937, an allogeneic B-cell line (DR8allo), and autologous E- cells at a 20:1 effector to target ratio. Data represent the mean percent specific ^{51}Cr released ± sem of triplicate assays.

Figure 3.1



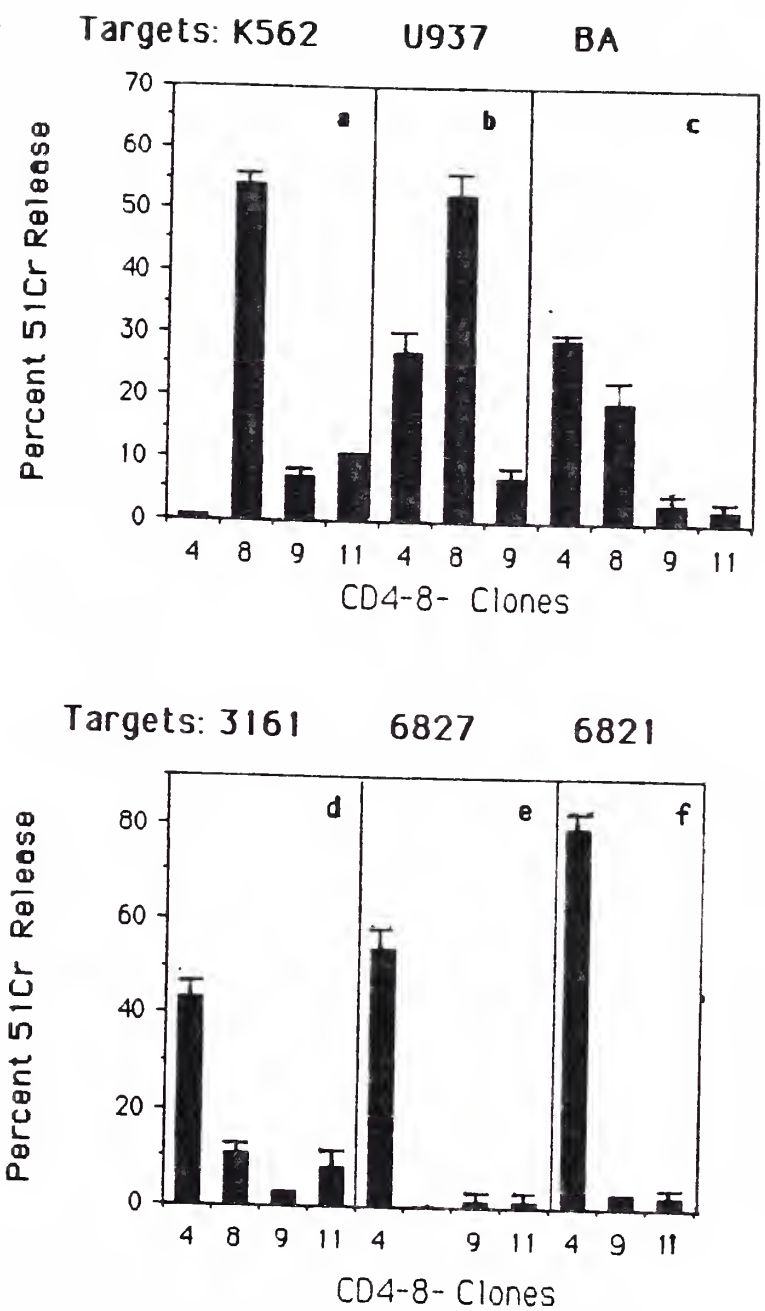
CD4-8- clones demonstrate heterogeneous cytotoxicity to K562 (top) and U937 (bottom) at a 20:1 effector to target ratio. A five hour ^{51}Cr release assays was performed in triplicate.

Figure 3.2



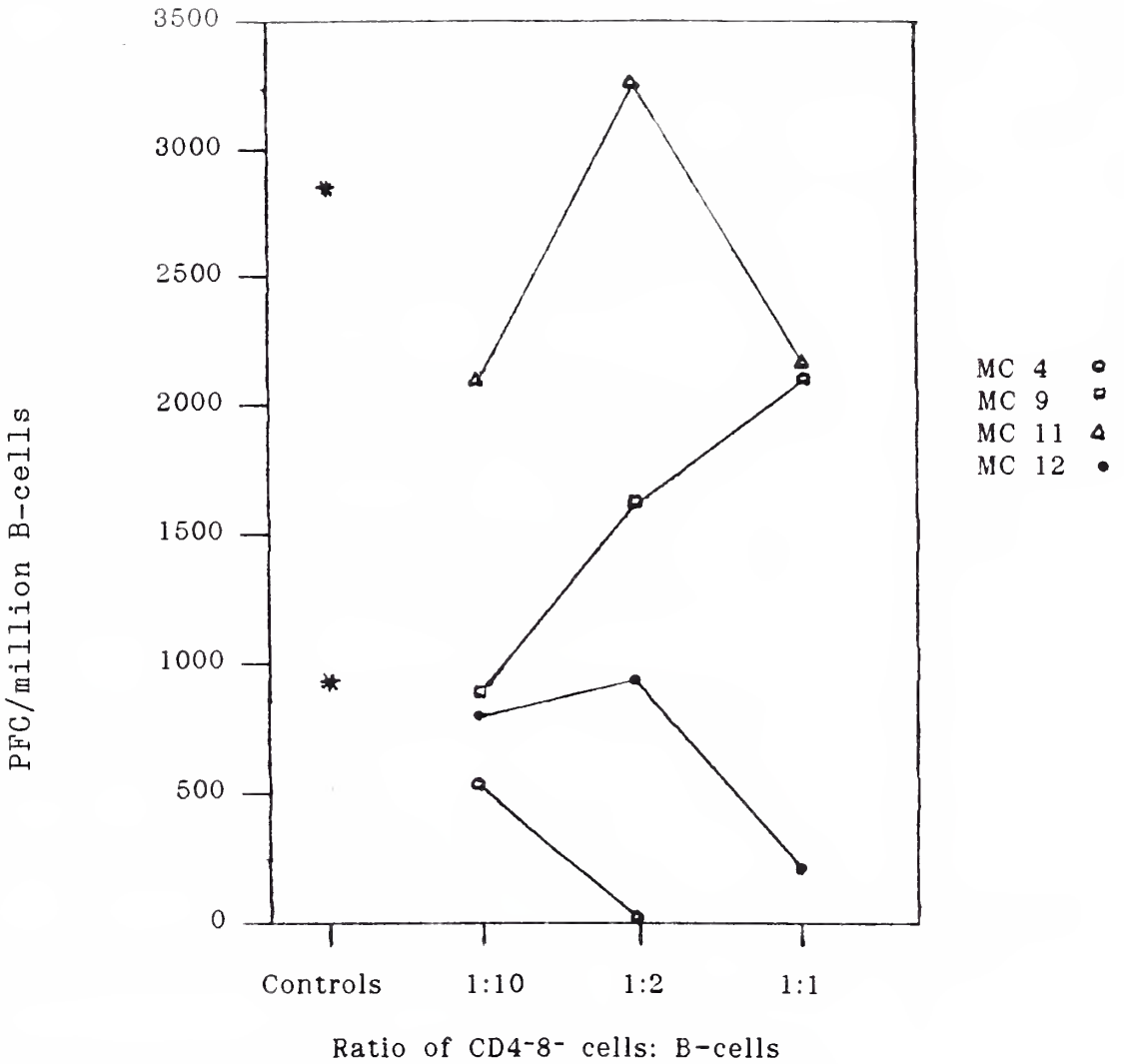
All MCs are cytotoxic to U937, and only MC4 is cytotoxic to BA, a B-cell line. $CD4^-8^-$ clones were incubated with ^{51}Cr labelled U937 (bottom) or BA (top) at a 20:1 e:t ratio. Data represent the mean of triplicate assays ± sem.

Figure 4.



MC4 alone, is cytotoxic to B-cells. CD4-8- T-cells were tested for cytotoxicity to a) K562, a standard NK target, b) U937, a monocyte, c) BA, a B-cell line, and B-cell lines of different DR specificities d) 3161, e)6827, and f)6821. Targets were labelled with ^{51}Cr and incubated at a 20:1 e:t ratio for 5 hours. Percent specific cytotoxicity is the mean ± sem of triplicate assays.

Figure 5 Inducer Function



MC cells exhibit heterogeneous helper function. $CD4^{-}8^{-}$ T-cells were added in ratios of 1:10, 1:2, and 1:1 to 0.25 x 10^6 autologous B-cells with PWM at 1:200 and plaque forming capacity (PFC) was assayed 7 days later. (See reverse hemolytic plaque assay in Materials and Methods). Controls were the B-cells with PWM 1:200 in medium (*) and in the presence of 100% that number of autologous E^{+} cells (*). Assays were performed in duplicate and the mean PFC/10^6 B-cells is recorded.

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